Genetic manipulation of the peptidolytic system in lactic acid bacteria

Kok, J; Venema, G

Published in:
International Dairy Journal

DOI:
10.1016/0958-6946(95)00029-1

Publication date:
1995

ELSEVIER

Int. Dairy Journal 5 (1995) 737–755

0958-6946/95/$9.50 + .00

0958-6946(95)00029-1

Genetic Manipulation of the Peptidolytic System in Lactic Acid Bacteria

Jan Kok* & Gerard Venema

Groningen Biomolecular Sciences and Biotechnology Institute, Department of Genetics, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

ABSTRACT

Due to their presumed involvement in product flavour the peptidases of lactic acid bacteria have been subject to extensive research. A major breakthrough was the isolation and purification of the various enzymes to homogeniety. This allowed a re-evaluation of the number of different enzymes in one species by careful investigation of their specificities. Moreover, the purified proteins offered novel ways to the genetic analysis of the peptidolytic system. N-terminal amino acid sequences of, and antibodies against, the purified peptidases were used to clone the majority of peptidases described for Lactococcus lactis. *Some peptidase genes, especially those from a number of lactobacilli, were obtained by complementation of peptidase-negative cloning hosts. Thus, by deduction, the amino acid sequences of these peptidases is known and all belong to known families of peptidases. The recently developed chromosomal integration systems for LAB have been used to construct (multiple) peptidase-negative mutants in* L. lactis. *The growth characteristics of these strains are now under study. As a number of the mutants were made in a food grade way their performance in cheesemaking can be evaluated. The review presented will focus on the latest developments in the genetic analysis of peptidases in LAB and will evaluate our current understanding of peptidolysis by LAB and its importance in dairy fermentations.*

INTRODUCTION

Many strains of lactic acid bacteria contain proteolytic systems that allow them to liberate essential and growth stimulatory amino acids and small peptides from the protein-rich substrates such as milk, meat and vegetables in which they are

*Author to whom all correspondence should be addressed.

primarily found. Centuries of selection on these and other properties of lactic acid bacteria have resulted in the availability of strains very well suited for the production of fermented foods and feed. Since the emergence of large scale industrial fermentation technologies, considerable stress has been placed on the reliable performance of starter strains resulting in an urge to know more about the molecular biology of these economically important microorganisms. The realisation that proteolysis by certain strains of lactic acid bacteria is an unstable trait, has led to a considerable research effort to try and understand the underlying mechanism, and has resulted in a detailed knowledge of the components involved in protein hydrolysis by these organisms. It is clear now that the system used to break down milk protein encompasses a proteinase that performs the initial cleavage of casein, a number of uptake systems that internalize the liberated peptides and amino acids, and an array of peptidases with different cleavage specificities degrading the large peptides into smaller peptides and, ultimately, into free amino acids.

The first step in proteolysis, the degradation of casein by the proteinases of lactic acid bacteria, has been dealt with extensively in recent reviews (Kok, 1990; Pritchard & Coolbear, 1993; Kok & de Vos, 1994).Only salient new aspects of this process will be dealt with in this overview. New insights into the processes of peptide uptake by lactococci have recently accumulated and will be reviewed here. The main body of this work, however, will be the evaluation of the peptidolytic activity of these organisms with emphasis on the genetic analysis of the enzymes involved. Moreover, the possibilities of genetically manipulating the system once the genes and regulatory elements are known will be discussed. As the latter aspect can at present only be fruitfully addressed in the lactococci, the peptidolytic system of these organisms will be dealt with most extensively. Space requirements preclude full citation of all the original literature. Most of these can be found in the review by Kok & de Vos (1994).

PROTEINASES

Proteinases from lactococci and lactobacilli used in milk fermentations are closely related. In both groups of organisms the proteinase involved in casein degradation is a cell envelope-located molecule that is initially produced as a preproprotein. The precursor is subject to self-cleavage resulting in the mature, active enzyme, a process for which the lipoprotein PrtM is essential. PrtM may be considered as an extracellular chaperone and shows similarities to a protein, PrsA of *Bacillus subtilis*, for which a comparable function has been postulated (for review, see Kok & de Vos, 1994).

Although it is an important component of yoghurt starter cultures used in dairy fermentations, the proteolytic system of *Streptococcus salvarius* ssp. *thermophilus* (*S. thermophilus*) has received much less attention than that of lactococci and lactobacilli. In general, *S. thermophilus* is proteolytically not very active. Among 97 strains of *S. thermophilus* tested only three possessed proteinase activity levels comparable to that of proteinase-positive strains of *L. lactis* (Shahbal *et al.*, 1991). The enzymes of two of these strains appeared refractory to purification (Shahbal *et al.*, 1993). Proteinase activity was not released from the cell wall by incubation of the cells in a Ca^{2+}-free buffer, a standard technique used to liberate lactococcal

proteinases, indicating that the enzyme is either tightly coupled to the cell wall or subject to rapid denaturation or (self-) digestion during the extraction procedure. The streptococcal proteinases are serine-type enzymes as they are inhibited by PMSF and DFP. The enzymes may be quite different from the lactococcal proteinase as a DNA probe of the lactococcal *prt* genes did not hybridise with chromosomal DNA of *S. thermophilus*. Moreover, antibodies specific for the *L. lactis* proteinase did not react with proteins from *S. thermophilus*.

The casein-degrading enzymes of Lactococcus and Lactobacillus are serine proteinases, the substrate specificity of which determines the nature of the degradation products liberated from casein and, thus, the growth of the organisms in milk and the generation of flavour compounds in the ultimate fermentation product. The importance in flavour development has resulted in a detailed analysis of the substrate specificity of the *L. lactis* proteinase. Two main specificities have been identified by analysing casein breakdown products by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). Proteinases of the P_I-type preferentially degrade β-casein and not, or only slightly, α_{s1}-casein. P_{III}-type proteinases degrade α_{s1}-and β-casein and the latter with a specificity that is distinct from that of the P_I-type proteinases (Visser *et al.*, 1986). The specificity of κ-casein breakdown has also been used to distinguish P_I-and P_{III}-type proteinases (Reid *et al.*, 1994). Studies addressing the peptide bond cleavage specificity of representatives of the two proteinase classes using SDS–PAGE have revealed that the lactococcal proteinases are able to liberate relatively small peptides from casein (Reid *et al.*, 1991; Visser *et al.*, 1991; Monnet *et al.*, 1992). A detailed analysis using reverse-phase high performance liquid chromatography (HPLC) combined with mass spectrometry indicated the liberation of numerous small peptides from casein by a P_I-type proteinase (Juillard *et al.*, 1995). About a hundred different peptides were identified but release of free amino acids other than phenylalanine, or di- and tripeptides was not observed. From this work it is clear that the P_I-type proteinase has a relatively broad specificity. About 17% of the peptides were composed of four to eight residues. These results emphasize the fact that, apart from the proteinase, *L. lactis* does not need extracellular peptidases to be able to grow in milk. The proteinase produces peptides that contain all the amino acids the cells need and a subset of the peptides are of the size that can be taken up by the oligopeptide transport system (see below). However, a strain producing the P_I-type proteinase is in short supply of the essential amino acids leucine and histidine. The results of Reid *et al.* (1994) suggest that at least the latter amino acid is provided by the histidine-rich peptides released early from κ-casein.

PEPTIDASES

The peptidolytic properties of lactic acid bacteria, most notably those of *L. lactis* and a number of Lactobacillus species, have been studied extensively. Starting with a biochemical analysis of the number of different peptidases in a single strain or species, the purification of the various enzymes to homogeneity and their detailed enzymatic characterization, the emphasis in this field has more recently shifted to the dissection of the genetic organization of the peptidolytic system.

Peptidases of *lactobacillus*

Table 1 lists the peptidases of various species of Lactobacillus for which the corresponding genes have been cloned and sequenced. Also indicated are those peptidases for which a defined mutation in the peptidase gene is available. It is clear from this compilation that our knowledge of the genetics of the peptidolytic system of lactobacilli is rapidly increasing. In the following, a brief description of the genetic data will be given.

Proline iminopeptidases
The genes of three proline iminopeptidases have been cloned and sequenced to date. The *Lactobacillus delbrueckii* ssp. *bulgaricus* (*Lb. bulgaricus*) proline iminopeptidase PepIP is a proline-specific peptidase capable of hydrolysing di- and tripeptides with proline at the N-terminal position, but not longer peptides

TABLE 1
Peptidases from Lactobacilli and *Streptococcus thermophilus* for which the proteins have been purified or the gene sequenced[a]

Organism	*Enzyme*[b]	*Monomer* M_r ($\times 10^{-3}$)[c]	*Class*	*Substrate*[d]	[e]
S. salivarius ssp. *thermophilus*	St-PepN	97	metallo	Lys-*p*NA	
	st-pepC	50	thiol	Lys-*p*NA Phe-βNA	+
Lb. delbrueckii ssp. *lactis*	PepN	95	metallo	Lys-βNA	+
	PepC	51	(thiol)[f]	Gly-Ala-βNA	+
	PepL	34	serine	Leu-βNA	+
	PepI	35	metallo	Pro-βNA	+
	PepV	52	metallo	β-Ala-His	+
	PepX	88	serine	Ala-Pro-*p*NA	+
Lb. delbrueckii ssp. *bulgaricus*	PepIP	33	serine	Pro-*p*NA	+
Lb. helveticus	PepC	49	(thiol)[f]	Lys-*p*NA	Y+
	PepN	93	metallo	Lys-*p*NA	Y+
	pepXP	88	(serine)[f]	Ala-Pro-*p*NA	Y+
	PepPN	35	(serine)[f]	Pro-Leu	+
	PepDI	54		Arg-Leu	Y+

[a] Literature references are given in the text.
[b] The genetic abbreviation is given where appropriate.
[c] Calculated from the genes when available.
[d] Routinely used substrate.
[e] +, gene cloned and sequenced; Y, knock-out mutant available.
[f] Based on amino acid sequence similarity.

(Atlan *et al.*, 1994; Gilbert *et al.*, 1994). PepIP has a deduced molecular mass of 33 kDa and forms trimers of M_r 100,000. The N-terminal amino acid sequence of the enzyme purified from an *E. coli* overproducer was the same as that of the primary translation product of *pepIP* indicating that PepIP does not undergo N-terminal processing in this host. Most probably, the enzyme has an intracellular location in *Lb. bulgaricus*. A very similar enzyme was found in *Lb. delbrueckii* ssp. *lactis* (*Lb. lactis*). The gene of this protein, *pepI*, has been cloned and sequenced recently (Klein *et al.*, 1994a). Only eight out of 294 and 295 amino acids that constitute PepI and PepIP, respectively, are different. The proline iminopeptidase from *Lb. helveticus*, designated PepPN, shows 32% similarity with the iminopeptidases of the two *Lb. delbrueckii* subspecies (Dudley & Steele, 1994). All three enzymes contain the motif GXSXGG specific for the active site of prolyl oligopeptidases and, accordingly, all three are inhibited by serine proteinase inhibitors.

Aminopeptidases

The pepN gene of *Lb. lactis* has been cloned by complementation of *E. coli* for the ability to hydrolyse His-β-naphthylamide (His-βNA) and Phe-βNA (Klein *et al.*, 1993). The deduced molecular mass of the lactobacillar PepN is 95 kDa and the enzyme is active as a monomer. It is a metallopeptidase belonging to the aminopeptidase N family and is most similar to PepN of *L. lactis* (62% similarity). Of a number of *p*-nitroanilide (*p*NA) substrates tested, Lys-*p*NA was cleaved best. *pepN* of *Lb. helveticus*, encoding the 97-kDa metalloaminopeptidase PepN, has been cloned and sequenced, and overexpressed in *L. lactis* (Nowakowski *et al.*, 1993; J.L. Steele, pers. comm.). The enzyme from *Lb. helveticus* showed 72% and 49% amino acid similarity to the PepN aminopeptidases from *Lb. lactis* and *L. lactis*, respectively.

Enzymes with similarity to the general aminopeptidase PepC of *L. lactis* have also been identified in lactobacilli. Recently, the nucleotide sequences of the *pepC* genes of *Lb. lactis* and *Lb. helveticus* became available (Klein *et al.*, 1994b; Fernández *et al.*, 1994). The deduced molecular masses of both enzymes are 50.9 and 48.8 kDa, respectively, and both show high (75%) amino acid sequence identity. The overall identities with lactococcal PepC are 50 and 54%, respectively. Two regions probably involved in catalysis were identified on the basis of homologies with the cysteine proteinase family exemplified by papain and, based on these similarities, the two Lactobacillus PepC's are also taken to be thiol aminopeptidases. Using chromogenic dipeptide-and amino acid substrates in an enzymatic plate assay, PepC from *Lb. lactis* was shown to be a broad-specificity aminopeptidase, hydrolysing any amino acid from the N-terminal except proline (Klein *et al.*, 1994b).

The gene of an enzyme from *Lb. lactis* with leucyl-aminopeptidase activity, PepL, has recently been cloned in a multiple peptidase-negative *E. coli* strain using Leu-βNA as the chromogenic substrate (Klein *et al.*, 1994c). Two peptidase genes were thus isolated: *pepN* described above and *pepL*, encoding a protein of 35 kDa. PepL showed homology to the prolinase PepPN of *Lb. helveticus* (46% identity) and the proline iminopeptidases of both *Lb. debrueckii* subspecies (25% identity). Moreover, significant similarities were observed with iminopeptidases of *Bacillus subtilis* (22% identity) and *Neisseria gonorrhoeae* (16% identical residues).

Dipeptidases
Lb. lactis can hydrolyse unusual β-alanyl-dipeptides such as β-Ala-His (carnosine). The gene encoding this activity, *pepV*, has been isolated from a gene bank of *Lb. lactis* in *E. coli* UK197 (Vongerichten *et al.*, 1994). This strain lacks *pepD* and *hisG* and, therefore, depends on a (cleavable) extracellular histidine source. *pepV*-positive clones were identified by their ability to grow on minimal plates supplemented with carnosine. The deduced enzyme, 470 amino acids with a calculated molecular mass of 52 kDa, showed similarity only with the dipeptidase PepV of *L. lactis* (47% identity over their entire lengths). Distant relationship-predicting computer programmes revealed homology of both enzymes to the ArgE/DapE/CPG2/YscS family of proteins that share the common characteristic of hydrolysing amide bonds in substrates with similar structure and a dependence on cobalt or zinc for activity. Interestingly, PepV was shown by inhibition studies to be a metallopeptidase.

The gene encoding the peptidase activity DPI identified by Nowakowski *et al.* (1993) has recently been sequenced (J.L. Steele, pers. comm.). The deduced amino acid sequence of DPI, with a calculated molecular mass of 54 kDa, did not show significant similarity to other known proteins.

X-prolyl dipeptidyl aminopeptidase
The gene for PepX of *Lb. lactis* was identified by a chromogenic plate assay in a plasmid bank of this species in *E. coli* (Meyer-Barton *et al.*, 1993). The nucleotide sequence revealed that PepX contains 792 amino acids with a calculated molecular mass of 88 kDa. The enzyme is not N-terminally processed, does not seem to contain transmembrane sequences, and is most probably an intracellular enzyme. PepX is 36% identical to the lactococcal PepXP's and shows 70% identity with PepXP of *Lb. helveticus*, for which the gene has recently been cloned and sequenced (Nowakowski *et al.*, 1993; J.L. Steele, pers. comm.)

Peptidases of *Streptococcus thermophilus*

Table 1 lists the enzymes that have been purified to homogeniety from *S. thermophilus*. There are indications that *S. thermophilus* contains a peptidolytic system that may be as complex as that of the lactococci (Chapot-Chartier *et al.*, 1994 and references therein). Genetic data on the system are now emerging.

Aminopeptidases
Two aminopeptidases, the streptococcal equivalents of PepN and PepC (st-PepN and st-PepC), have been analysed in quite some detail recently.

st-PepN has been independently isolated to homogeniety by two groups (Midwinter & Pritchard, 1994; Rul *et al.*, 1994). Both groups used Lys-*p*NA as the substrate during purification. The enzyme is a metallopeptidase with a broad substrate specificity towards *p*NA amino acids derivatives. Di- and tripeptides are also hydrolysed, with a preference for hydrophobic or basic amino acids at the N-terminal position, but the enzyme shows a preference for longer peptides. It successively removed amino acids from the N-terminal of various oligopeptides but did not cleave peptide bonds on the N-terminal side of a proline residue. The molecular size of st-PepN is 97 kDa and, despite the slight discrepancy in the molecular masses, the PepN activity isolated by Tsakalidou & Kalantzopoulos

(1992) may in fact represent the same enzyme. The enzyme is most probably located intracellularly. The N-terminal sequence of 21 amino acids of the mature enzyme shows a fairly high degree of similarity with that of PepN of *L. lactis*.

The only peptidase gene of *S. thermophilus* cloned and sequenced thus far is that of st-PepC (Chapot-Chartier *et al.*, 1994). The gene has been cloned by complementation of an *E. coli* strain for the ability to degrade Phe-βNA. st-PepC is highly similar to PepC from *L. lactis* (70% identity) and, like the latter, contains a region with strong similarity to the active site of cysteine proteinases. The bacterial PepC-type enzymes and the eukaryotic bleomycin hydrolase appear to constitute a new family of thiol aminopeptidases. st-PepC has a molecular mass of 50.4 kDa and seems to be an intracellular enzyme considering the fact that it is not N-terminally processed. Overproduction in *E. coli* allowed purification and biochemical characterization of the enzyme. St-PepC exists in a hexameric structure with a mass of 300 kDa and has a broad substrate specificity.

Peptidases from *Lactococcus lactis*

In Table 2, the latest details of the peptidases purified and/or cloned from Lactococcus are compiled. All enzymes listed in this table attack peptides from the N-terminal end. To date, no carboxypeptidase activity has been described in *L. lactis*. In total some fourteen peptidases have been studied in detail and will be discussed briefly here. For a more detailed description the reader is recommended to read the original literature given in the reference list or the recent review by Kok & de Vos (1994).

Aminopeptidases

Aminopeptidases are common in lactococci and have been purified to homogeniety from various strains of *L. lactis*. They can be roughly divided into two classes, the general aminopeptidases PepA, PepC, PepN, and PCP, and the proline-specific peptidases such as PepXP, prolidase, and proline iminopeptidase. Apart from the latter two, the genes of all of the other peptidases have been cloned and sequenced. All general aminopeptidases have neither endopeptidase nor caboxypeptidase activity. All are inhibited by sulfhydryl-blocking agents and EDTA and are, thus, metalloenzymes. PepC is an exception as it is blocked by chemicals reacting with thiol groups.

General aminopeptidases

The gene of PepN, an aminopeptidase with broad substrate specificity, has been sequenced from two strains of *L. lactis* ssp. *cremoris* and the two sequences were shown to be almost identical (Tan *et al.*, 1992; Strøman, 1992). PepN was given its name because of its similarities to aminopeptidase N of both eu-and prokaryotic origin. The enzyme has a deduced molecular mass of 95.4 kDa. Of six regions in the amino acid sequence of *L. lactis* PepN which show similarity to other aminopeptidase N sequences, one region (comprising residues 281 to 301) shows significantly higher sequence similarity and may contain the active site of PepN. This region shows homology to a segment in Zn-dependent neutral proteinases of the thermolysin family that is part of the active site and contains an essential Zn-ion binding site.

TABLE 2
Lactococcal peptidases[a]

Enzyme[b]	*Purified*	*Monomer* M_r $(\times 10^{-3})$[c]	*Class*	*Substrate*[d]	*Gene sequenced*
PrtP	Y[e]	200	serine	casein	Y
neutral proteinase		93	neutral	β-casein	
PepA	Y	39	metallo	Glu/Asp-*p*NA	Y
PepC	Y	48	thiol	Leu/Lys-*p*NA	Y
PepN	Y	95	metallo	Leu/Lys-*p*NA	Y
PepXP	Y	88	serine	X-Pro-*p*NA	Y
amino-peptidase P	Y	44	metallo	bradykinin	
PCP	—	25	serine	pyroGlu-*p*NA	Y
PepV	Y	49	metallo	Leu-Leu	Y
PepT	Y	52	metallo	tripeptides	Y
prolidase	Y	43	metallo	X-Pro	
proline imino peptidase	Y	50	metallo	Pro-X-(Y)	
PepF[f]	Y	70	metallo	bradykinin	Y
PepO[g]	Y	70	metallo	Metenkephalin	Y

[a] Literature references are given in the text.
[b] The genetic abbreviation is given where appropriate.
[c] Calculated from the genes when available.
[d] Routinely used substrate.
[e] Y = yes.
[f] PepF is identical to LEP I.
[g] PepO and NOP are the same enzymes.

PepC is a general aminopeptidase present in various strains of lactococci, albeight at different levels of activity. The gene was cloned from *L. lactis* ssp. *cremoris* strain AM2 by complementation of *E. coli* (*pepN*) (Chapot-Chartier *et al.*, 1992). As with PepN, PepC was given its name on the basis of the genetic study, indicating similarity of the enzyme to cysteine proteinases such as papain and aleurin. A marked difference between PepC and the other cysteine proteinases is that the latter are about half the size of PepC. Two regions encompassing the amino acids of the active centre of papain are especially well conserved and may in fact form the active site of PepC. This would agree with the observation that PepC is inhibited by thiol-reacting reagents.

The genes of two narrow specificity aminopeptidases, glutamyl aminopeptidase (PepA) and pyrrolydonyl carboxylyl peptidase (PCP), have recently been isolated and characterized. The *pepA* gene was cloned by employing the N-terminal amino acid sequence of the purified enzyme (M.J. Gasson, pers. comm.). The deduced protein has a molecular mass of 38.1 kDa and is, thus, somewhat smaller than that determined previously by biochemical techniques. The enzyme resembles mammalian aminopeptidase A in that it is highly specific for glutamyl-and

aspartylpeptides (hence the name PepA). It shows homology with an endoglucanase of *Clostridium thermocellum*.

The gene for PCP was cloned by polymerase chain reaction techniques using conserved amino acid sequences of the PCPs of *Bacillus subtilis* and *Streptococcus pneumonia* (A.J. Haandrikman, pers. comm.). The deduced molecular size of lactococcal PCP is 25 kDa and, not surprizingly, the enzyme shows similarity to its bacillar and streptococcal counterparts.

Proline-specific peptidases

PepXP, X-prolyl dipeptidyl aminopeptidase, is, as its name indicates, able to cleave X-Pro dipeptides from the N-terminal of oligopeptides. It is the only proline-specific peptidase of Lactococcus to date for which the gene has been cloned and sequenced. The deduced amino acid sequences of the genes cloned from two *L. lactis* ssp. *cremoris* strains were almost identical and confirmed earlier biochemical data on the size of the enzymes: 88 kDa or 763 amino acids (Mayo *et al.*, 1991; Nardi *et al.*, 1991). The enzyme does not show significant similarity to other proteins. Recently, the active site of PepXP has been identified by [^{3}H]DFP labeling (Chich *et al.*, 1992). The active site of PepXP showed only limited homologies with other serine proteinases but the motif GXSYXG was present in all three known X-prolyl dipeptidyl aminopeptidases (PepXP, mammalian DPPIV and yeast DPAB). The presence of a reactive serine residue in the active site fits with inhibitor studies showing that PepXP is inactivated by serine proteinase inhibitors.

Prolidase of *L. lactis* H61 has a specificity that would perfectly complement the action of PepXP: it only degrades X-Pro dipeptides (Kaminogawa *et al.*, 1984). The protein has a molecular size of 43 kDa.

A proline iminopeptidase of approximately 50 kDa has been purified from *L. lactis* HP (Baankreis & Exterkate, 1991). It was shown to be present in all lactococcal strains tested but not in lactobacillus species. The enzyme hydrolyzes almost all di-and tripeptides with Pro as the amino terminal residue but does not cleave X-Pro bonds or peptides longer than four residues.

Recently, aminopeptidase P was purified to homogeniety from *L. lactis* NCDO 763 by using bradykinin (RPPGFSPFR) as the substrate (Mars & Monnet, 1994). The aminopeptidase is a metalloenzyme with a molecular size of 44 kDa. It is highly specific for X-Pro-Pro N-terminal sequences and does not exhibit prolidase-like activity on X-Pro dipeptides. The optimal size of the substrate seems to be near five residues.

Dipeptidases and tripeptidases

Recently, dipeptidase genes have been cloned and sequenced from two *L. lactis* ssp. *cremoris* strains (P. Strøman & F. Mulholland, pers. comm). The deduced amino acid sequences of the enzymes, approximately 52 kDa in size, differed in 18 out of the 473 amino acid residues. PepV, as the enzyme was designated, showed similarities to the carnosinase of *Lb. lactis* described above and to *E. coli* succinyl-diaminopimelate desuccinylase and acetylornithine deacetylase, two enzymes that cleave a peptide bond-like site in their respective substrates. Moreover, these enzymes are similar to carboxypeptidase G2, a Zn-metallopeptidase.

The gene of the tripeptidase PepT of *L. lactis* ssp. cremoris Wg2 has been cloned by reverse genetics (Mierau *et al.*, 1994). The *pepT* gene may be in an

operon with two ORFs of unknown function surrounding it, as no promoter nor terminator structures could be identified immediately upstream or downstream of *pepT*. PepT contains 413 amino acids and the deduced molecular size, 46 kDa, is somewhat smaller than that determined biochemically (52 kDa). PepT shows significant amino acid sequence similarity with PepT of *Salmonella typhimurium* and carboxypeptidase G of *Pseudomonas putida*.

Endopeptidases

Several reports exist on the purification of oligopeptidases from lactococci but, on the basis of their substrate specificities, only two different enzymes seem to be present in these organisms (Monnet, 1994). PepO, purified to homogeniety using Met-enkephalin as the substrate, was the first endopeptidase for which the gene was isolated and sequenced (Mierau *et al.*, 1993). The gene is immediately downstream of the oligopeptide permease operon, *opp* (see below) and is transcribed from a promoter upstream of *oppA*, the last gene of opp. PepO contains 627 amino acid residues and has a calculated molecular mass of 71.5 kDa. The enzyme shows striking similarities to the mammalian neutral endopeptidase, enkephalinase, especially in a segment of 42 amino acids containing the putative active site of the protein and a motif (HEXXH) characteristic for zinc-dependent metalloproteinases.

PepF was purified using the fact that it specifically cleaves the F—S bond in bradykinin as a distinctive marker (Mars & Monnet, 1994). The enzyme cleaves peptides containing seven to seventeen amino acid residues, not smaller or larger peptides, while the cleavage site should be at least three residues from the C-terminal of the substrate. The *pepF* gene was located on the lactose-proteinase plasmid of *L. lactis* NCDO 763 and was cloned by reverse genetics and PCR strategies. PepF contains 601 amino acids with a deduced molecular size of 70 kDa (Monnet *et al.*, 1994). The characteristic Zn-dependent metalloproteinase motif HEXXH was present in position 387 to 391 of the enzyme. PepF shows homology with a number of bacterial metallopeptidases sharing this motif, of which dipeptidyl carboxypeptidase of *E. coli* shows the highest identity. A second region of 33 amino acids shows similarity with creatine kinases from vertebrates and with arginine kinase from lobster.

AMINO ACID AND PEPTIDE TRANSPORT SYSTEMS

To date, none of the amino acid carriers described in detail biochemically (for review, see Konings *et al.*, 1989) have been characterized genetically. A system devoted to the internalization of di-and tripeptides and a carrier involved in oligopeptide uptake have been analysed biochemically and genetically relatively recently and will be discussed here.

Di-and tripeptide uptake

Di-and tripeptide uptake by lactococci is a proton-motive-force-driven process and the system has a broad substrate specificity (Smid *et al.*, 1989a). It has a high specificity for proline-containing dipeptides, a property which was used to clone the gene involved by complementation of a dipeptide transport-negative *proC*

mutant of *E. coli* (Hagting *et al.*, 1994). The di- and tripeptide transporter, DtpT, appeared to be specified by a single gene encoding an integral membrane protein with 12 putative transmenbrane sequences. It contains 463 amino acid residues and has a molecular size of 50.6 kDa. So far, DtpT of *L. lactis* is the only proton-linked peptide transport system described in bacteria.

Oligopeptide transport

Oligopeptides are taken up by *L. lactis* via an ATP-driven transport system (Opp) that is capable of transporting peptides of four to at least eight amino acid residues (Kunji *et al.*, 1993; Tynkkynen *et al.*, 1993). As discussed above, β-casein is degraded by PrtP to peptide fragments of four to thirty residues, of which 17% are smaller than nine residues and, thus, potential substrates for Opp. Indeed, all essential and growth-stimulating amino acids for *L. lactis* are released from β-casein in the form of peptides that are internalized exclusively by Opp at rates high enough to meet the growth requirements. Only His and Leu had to be added to a chemically defined medium containing β-casein as the protein substrate to allow good growth (Kunji *et al.*, 1995). Inactivation of Opp led to a complete block in oligopeptide uptake and growth in milk or on β-casein.

The five genes encoding the oligopeptide transport system of *L. lactis* have been cloned and sequenced (Tynkkynen *et al.*, 1993). Opp belongs to the superfamily of ABC transporters. This family encompasses, among others, all bacterial peptide transport systems described so far, with the exception of the lactococcal DtpT (see above). Two genes, *oppB* and *oppC*, encode the transmembrane components of the system, *oppD* and *oppF* specify the two proteins that are thought to deliver the energy for transport, while *oppA* codes for the lipoprotein OppA, the substrate-binding protein.

MANIPULATING THE PEPTIDOLYTIC SYSTEM

There are several reasons to manipulate the proteolytic and peptidolytic systems of lactic acid bacteria. Firstly, there is a fundamental interest as the availability of mutants in these systems would make it possible to decipher their complexity. Then, the mutants would be very helpful in assessing the importance of the product of a mutated gene(s) in industrial processes. Ultimately, this knowledge would permit the engineering of biochemical properties and product levels of individual components of the proteolytic system in a single strain and in a mixture of strains used in starter blends.

Manipulation of *prt*

The first step in casein breakdown by *L. lactis* has been manipulated in various ways. As this has been reviewed recently (Kok & de Vos, 1994), it will only be discussed briefly here. Strains lacking proteinase PrtP activity were obtained either spontaneously, as a consequence of proteinase plasmid loss or deletion formation, or have been made by *in vitro* genetic mutation of active site residues (for reviews, see Kok, 1990; Pritchard & Coolbear, 1993; Kok & de Vos, 1994). These studies have revealed that PrtP is essential for lactococci to grow in milk.

Also, the copy number of the *prt* genes has been steered at will by combining them on a plasmid with a copy number higher than the original proteinase plasmid or by placing them on the chromosome of *L. lactis*. In the latter case, the stability of the trait was shown to be dramatically increased (Leenhouts *et al.*, 1991). The specificity of the lactococcal proteinase has been engineered by constructing hybrids between P_I and P_{III}-type proteinases (Vos *et al.*, 1991). This and subsequent studies have identified regions determining the caseinolytic specificity of the enzyme and, moreover, led to the production of proteinases with novel specificities. In an entirely different approach, Van de Guchte *et al.* (1990) expressed the gene for neutral proteinase of *B. subtilis* in *L. lactis*.

Mutations in peptide uptake

A di-and tripeptide transport deficient mutant has been isolated by selection of *L. lactis* ML3 for resistance to the toxic dipeptide L-alanyl-β-chloro-L-alanine (DiACA), a substrate of this transport system (Smid *et al.*, 1989b). The mutant had normal proteinase and peptidase activities, was unaffected in the accumulation of peptides larger than three residues, but did not grow on a chemically defined medium with casein as the sole nitrogen source. The mutants still possessed residual transport activity for some di-and tripepides. Recently, a defined mutant in di-and tripeptide uptake was made. DtpT was deleted from the chromosome via homologous recombination (Hagting *et al.*, 1994).

A defined integration mutant in *oppA* was constructed and used to show that the oligopeptide transport system is essential for growth of *L. lactis* on peptides longer than three residues (Tynkkynen *et al.*, 1993). In this study it was shown that without a functional Opp system lactococci are not able to grow in milk. Moreover, the studies of Tynkkynen *et al.* (1993) and Kunji *et al.* (1993) clearly show that peptidases hydrolysing the casein breakdown products delivered by PrtP are all located intracellularly in *L. lactis*.

Construction of peptidase mutants

Peptidase-negative mutants of lactic acid bacteria have been obtained by mutagenizing strains with chemicals such as ethyl methane sulphonate and nitrosoguanidine. Studies in *Lb. bulgaricus* using two of such mutants, one affected in PepXP activity, the other one mutated in lysyl-aminopeptidase, have shown that neither is essential for growth of *Lb. bulgaricus* in milk (Atlan *et al.*, 1990). Some evidence was obtained for the existance of a common regulatory mechanism controlling the biosynthesis of th wo peptidases and proteinase activity.

A drawback of chemical mutagenesis is that one can never exclude the possibility that, apart from the peptidase gene under study, other parts of the chromosome have been mutated which may or may not directly influence the properties of the strain as far as performance in milk fermentations is concerned. Moreover, only a limited number of peptidases, namely those for which a good selection for mutants exists, will be easily accessible for analysis. This is reflected in the fact that until now chemical mutants in Lactobacillus have been isolated only for PepXP and lysyl-aminopeptidase activity. To circumvent these problems a number of groups have used the availability of the genes of several peptidases to make defined chromosomal knock-out mutations. The first example of such a

mutant was *L. lactis* (*pepXP*) (Mayo *et al.*, 1993). Analysis of this mutant showed that PepXP is not essential for growth in milk. Altered degradation of a peptide substrate was observed by an extract of the *pepXP* strain, suggesting that changing the expression of PepXP in a starter strain may have an impact on the peptide composition of fermented milk. In *Lb. helveticus* single and double mutants in *pepC*, *pepN* and *pepXP* have been constructed as well as a *pepC-pepNpepXP* triple mutant. Moreover, a deletion was made in the chromosomal gene for DPI. These strains are now being analysed with respect to growth in milk and rate of acid production (Bhowmik *et al.*, 1993; J.L. Steele, pers. comm.).

A two-step recombination procedure specifically developed for the easy selection of mutants in any gene, even ones of which the products are difficult to assay, has recently been developed. The strategy, depicted in Fig. 1, ultimately results in a gene replacement mutant that is devoid of foreign DNA (Leenhouts & Venema, 1993). Using this method, a series of isogenic peptidase mutants have been made in *L. lactis* MG1363 (I. Mierau, pers. comm.). A total of sixteen mutants including single, double, triple, quadruple mutants and one quintuple *pep* mutant are available (see Table 3). The *prt* genes of the parental strain *L. lactis* NCDO712 were introduced in each mutant in order to be able to study its growth and acidification rates in milk. Also, as a preliminary characterization the peptidase profiles of all strains have been examined. All strains except for two of the three four-fold mutants and the one quintuple mutant were able to grow in milk (Table 3). In the case of two mutants (*pepCpepN* and *pepOpepN*) a slight reduction of approximately 25% was observed in the final pH and optical density of the cultures after 24 h of growth at 30°C. The three strains that did not grow in milk performed even worse than a genuine Prt^- strain i.e. a strain lacking active proteinase. From these studies a number of conclusions can be drawn. First, they show that the peptidase system of *L. lactis* is a complex of peptidases with overlapping specificities. Moreover, it is likely that the diversity of degradation products generated by the proteinase and internalised by Opp allows various combinations of peptidases to liberate the essential amino acids. Only if every possible degradation pathway is blocked, as is apparently the case in the five-fold mutant, is growth in milk impeded. The fact that the growth of the quadruple and quintuple mutants is even less than a Prt^- strain indicates that residual growth of the Prt^- strain is for a large part attributable to the presence of free peptides in milk.

CHEESE TRIALS USING MUTANT OR RECOMBINANT LACTIC ACID BACTERIA

Experimental cheeses using either foodgrade (chemical) mutants of LAB or strains of lactococci that had been engineered genetically have recently been made and scrutinized.

The effect of two chemically-induced mutations, *pepN* and *pepXP*, on flavor development in Gouda-type cheeses has been examined by Baankreis (1992). The amount of mutant cells in the starter blend of 10% bitter-producing Prt^+ and 90% Prt^- cells was varied and the organoleptic quality and amino acid and salt-soluble nitrogen were examined. Increasing the concentration of the *pepXP* strain did not affect bitterness but resulted in a decrease in organoleptic quality. Raising

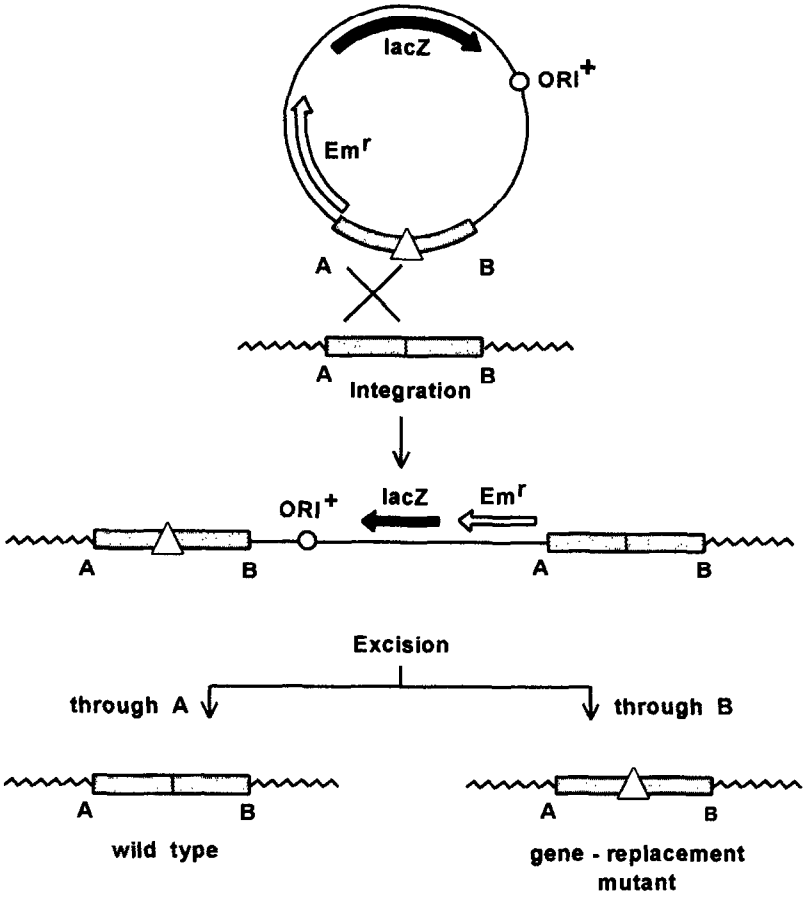

Fig. 1 Replacement recombination strategy using pORI280, a vector that cannot replicate in a host strain without the plasmid replication protein. ORI^+: plus origin of replication; A,B: 5'- and 3'-ends of gene to be mutated; ⌂: internal deletion in gene A/B. For details, see Leenhouts & Venema (1993), from which this figure is an adaptation.

the concentration of the *pepN* mutant led to a pronounced increase in bitterness which was attributed to the accumulation of low molecular weight bitter peptides.

A *Lb. casei* ssp. *casei* DAP (PepXP) mutant has been used to study the role of the enzyme in cheddar cheese ripening (El Abboudi *et al.*, 1992). To cheese milk which had been inoculated with a commercial cheese starter freeze-shocked *Lb. casei* was added just prior to renneting. No differences in cheese ripening and flavor development were observed between the wild-type and DAP-negative *Lb. casei* strains.

The effect on ripening and flavor development in cheddar cheese of genetically modified *L. lactis* has been recently assessed (McGarry *et al.*, 1994). Three-fold overproduction of PrtP did not enhance the quality of the cheese nor did it accelerate ripening. Cheeses made with strains producing the *B. subtilis* neutral

TABLE 3
Growth in milk of genetically defined *L. lactis* MG1363 peptidase mutants

Peptidase mutation[a]	*Milk clotting*[b]
X	+
O	+
T	+
C	+
N	+
XT	+
XN	+
XO	+
CN	+
ON	+
XTO	+
XTN	+
XTOC	+
XTON	−
XTNC	−
XTOCN	−

[a] X, T, O, C, N: PepXP-, PepT-, PepO-, PepC, PepN-negative, respectively. All strains carry the lactose/proteinase plasmid pLP712.
[b] Measured after 24 h at 30°C.

proteinase scored very poorly with respect to body and texture, although their flavor was satisfactory. α_{s1}-and β-casein in these cheeses were degraded very rapidly and were almost completely hydrolysed after one month of ripening. Clearly, such a powerful proteolytic activity is not desirable. By carefully controlling the amount of recombinant Npr-producing lactococcal cells in a blend with a wild-type starter strain a superior cheese was obtained that had fully ripened after two months (Fitzgerald, pers. comm.).

CONCLUSIONS AND PROSPECTS

The genes of many components of the proteolytic system of lactic acid bacteria have been cloned and analysed at the nucleotide sequence level. The last few years have seen a rapid increase in the number of peptidase genes cloned and sequenced from both lactococci and lactobacilli. In the lactococci, moreover, two peptide uptake systems, one for di-and tripeptides and the other one for oligopeptides, have been biochemically described and genetically dissected. Due to the development of genetic strategies that allow access to the chromosome of lactic acid bacteria it is now possible to specifically target and mutate the various genes involved in proteolysis in order to answer the following questions:

— is the gene under study essential for growth in milk?
— What is the impact of the mutation(s) on cheese ripening and flavor development?

Through the analysis of mutants we have now learned that the proteolytic system of lactococci is an intricate system in which the cell envelope-associated proteinase degrades casein into oligopeptides of four and more residues. The oligopeptide transport system is able to take up peptides with four to at least eight residues and a selection of the oligopeptides produced extracellularly by PrtP are internalized by Opp. These peptides are then further degraded by a complex of intracellular peptidases. Most probably as a result of the overlapping specificities of these peptidases and because essential amino acids will be available as components of different peptides in the diversity of internalized oligopeptides, all of the single, double, and triple *pep* mutants are able to grow in milk. The latter observation is very important as this will make it possible to analyse the effect of the mutations and, thus, by inference, the impact of the corresponding peptidases on cheese ripening and flavor development. This is the next crucial step to be taken in the process of understanding the intricacies of the proteolytic system of lactic acid bacteria to such an extent that rational modification of this system for the improvement of food products will ultimately be feasible. The set of *L. lactis* mutants compiled in Table 3 provides a good tool to start such an analysis and is especially interesting in this respect as the mutants have been constructed in such a way that they do not contain any foreign DNA and should, therefore, be considered 'foodgrade'.

ACKNOWLEDGEMENTS

We are grateful to Igor Mierau and Edmund Kunji for valuable discussions and would sincerely like to thank our colleagues for providing data prior to publication. Henk Mulder prepared the figure. Part of this work was supported by the Royal Netherlands Academy of Arts and Sciences (KNAW).

REFERENCES

Atlan, D., Laloi, P. & Portalier, R. (1990). X-prolyl dipeptidyl aminopeptidase of *Lactobacillus delbrueckii* subsp. *bulgaricus*: characterization of the enzyme and isolation of deficient mutants. *Appl. Environ. Microbiol.*, **56**, 2174–9.

Atlan, D., Gilbert, C., Blanc, B. & Portalier, R. (1994). Cloning, sequencing and characterization of the pepIP gene encoding a proline iminopeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397. *Microbiol.*, **140**, 527–35.

Baankreis, R. & Exterkate, F. A. (1991). Characterization of a peptidase from *Lactococcus lactis* ssp. *cremoris* HP that hydrolyses di-and tripeptides containing proline or hydrophobic residues as the aminoterminal amino acid. *System. Appl. Microbiol.*, **14**, 317–23.

Baankreis, R. (1992). The role of lactococcal peptidases in cheese ripening, Thesis, University of Amsterdam, Amsterdam, The Netherlands.

Bhowmik, T., Fernandez, L. & Steele, J. L. (1993). Gene replacement in *Lactobacillus helveticus*. *J. Bacteriol.*, **175**, 6342–4.

Chapot-Chartier, M.-P., Nardi, M., Chopin, M.-C., Chopin, A. & Gripon, C. (1992). Cloning and sequencing of pepC, a cysteine aminopeptidase gene from *Lactococcus lactis* subsp. *cremoris* AM2. *Appl. Environ. Microbiol.*, **59**, 330–3.

Chapot-Chartier, M.-P., Rul, F., Nardi, M. & Gripon, J.-C. (1994). Gene cloning and characterization of PepC, a cysteine aminopeptidase from *Streptococcus thermophilus*, with sequence similarity to the eucaryotic bleomycin hydrolase. *Eur. J. Biochem.*, **224**, 496–506.

Chich, J. F., Chapot-Chartier, M.-P., Ribadeau-Dumas, B. & Gripon, J.-C. (1992). Identification of the active site serine of the X-prolyl dipeptidyl aminopeptidase from *Lactococcus lactis*. *FEBS Lett.*, **314**, 139–42.

Dudley, E. G. & Steele, J. L. (1994). Nucleotide sequence and distribution of the pepPN gene from *Lactobacillus helveticus* CNRZ 32. *FEMS Microbiol. Lett.*, **119**, 41–6.

El Abboudi, M., El Soda, M., Johnson, M., Olson, N. F., Simard, R. E. & Pandian, S. (1992). Peptidase deficient mutants — a new tool for the study of cheese ripening. *Milchwissenschaft*, **47**, 625–8.

Fernández, L., Bhowmik, T. & Steele, J. L. (1994). Characterization of the *Lactobacillus helveticus* CNRZ32 pepC gene. *Appl. Environ. Microbiol.*, **60**, 333–6.

Gilbert, C., Atlan, D., Blanc, B. & Portalier, R. (1994). Proline iminopeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397: purification and characterization. *Microbiology.*, **140**, 537–42.

Hagting, A., Kunji, E. R. S., Leenhouts, K. J., Poolman, B. & Konings, W. N. (1994). The di-and tripeptide transport protein of *Lactococcus lactis*. *J. Biol. Chem.*, **269**, 11391–9.

Juillard, V., Laan, H., Kunji, E. R. S., Jeronimus-Stratingh, C. M., Bruins, A. P. & Konings, W. N. (1995). The extracellular P_I-type proteinase of *Lactococcus lactis* hydrolyses β-casein into more than one hundred different oligopeptides. *J. Bacteriol.*, **177**, 3472–8.

Kaminogawa, S., Azuma, N., Hwang, I. K., Suzuki, Y. & Yamauchi, K. (1984). Isolation and characterization of a prolidase from *Streptococcus cremoris* H61. *Agric. Biol. Chem.*, **48**, 3035–40.

Klein, J. R., Klein, U., Schad, M. & Plapp, R. (1993). Cloning, DNA sequence analysis and partial characterization of pepN, a lys-aminopeptidase from *Lactobacillus delbrueckii* ssp *lactis* DSM 7290. *Eur. J. Biochem.*, **217**, 105–14.

Klein, J. R., Schmidt, U. & Plapp, R. (1994a). Cloning, heterologous expression, and sequencing of a novel proline iminopeptidase gene, pepI, from *Lactobacillus delbrueckii* subsp. *lactis* DSM 7290. *Microbiology*, **140**, 1133–9.

Klein, J. R., Henrich, B. & Plapp, R. (1994b). Cloning and nucleotide sequence analysis of the *Lactobacillus delbrueckii* subsp. *lactis* DSM 7290 cysteine aminopeptidase gen pepC. *FEMS Microbiol. Lett.*, in press.

Klein, J. R., Dick, A., Schick, J., Matern, H. T., Henrich, B. & Plapp, R (1994c). Molecular cloning and DNA sequence analysis of pepL, a leucyl aminopeptidase from *Lactobacillus delbrueckii* subsp. *lactis* DSM 7290. *Eur. J. Biochem.*, in press.

Kok, J. (1990). Genetics of the proteolytic system of lactic acid bacteria. *FEMS Microbiol. Rev.*, **87**, 15–42.

Kok, J. & de Vos, W. M. (1994). The proteolytic system of lactic acid bacteria. In *Genetics and biotechnology of lactic acid bacteria*, eds, M. J. Gasson & W.M. de Vos, Blackie Academic and Professional, Glasgow, U.K., pp. 169–210.

Konings, W. N., Poolman, B. & Driessen, A. J. M. (1989). Bioenergetics and solute transport in lactococci. *CRC Critical Reviews in Microbiology*, **16**, 419–76.

Kunji, E. R. S., Smid, E. J., Plapp, R., Poolman, B. & Konings, W. N. (1993). Ditripeptides and oligopeptides are taken up via distinct transport mechanisms in *Lactococcus lactis*. *J. Bacteriol.*, **175**, 2052–9.

Kunji, E. R. S., Hagting, A., de Vries, C. J., Juillard, V., Haandrikman, A. J., Poolman, B. & Konings, W. N. (1995). Transport of β-casein-derived peptides by the oligopeptide

transport system is a crucial step in the proteolytic pathway of *Lactococcus lactis*. *J. Biol. Chem.*, **270**, 1569–74.

Leenhouts, K. J., Kok, J. & Venema, G. (1991). Chromosomal stabilization of the proteinase genes in Lactococcus lactis. *Appl. Environ. Microbiol.*, **57**, 2568–75.

Leenhouts, K. J. & Venema, G. (1993). Lactococcal plasmid vectors. In *Plasmids, A Practical Approach*, ed., K.G. Hardy. Oxford University Press, New York.

Mars, I. & Monnet, V. (1994). An aminopeptidase P from *Lactococcus lactis* with original specificity. *Biochem. Biophys. Acta*, **1243**, 209–15.

Mayo, B., Kok, J., Venema, K., Bockelmann, W., Teuber, M., Reinke, H. & Venema, G. (1991). Molecular cloning and sequence analysis of the X-prolyl dipeptidyl aminopeptidase gene from *Lactococcus lactis* subsp. *cremoris*. *Appl. Environ. Microbiol.*, **57**, 38–44.

Mayo, B., Kok, J., Bockelmann, W., Haandrikman, A., Leenhouts, K. J. & Venema, G. (1993). The effect of X-prolyl dipeptidyl aminopeptidase deficiency in *Lactococcus lactis*. *Appl. Environ. Microbiol.*, **59**, 2049–55.

McGarry, A., Law, J., Coffey, A., Daly, C., Fox, P. F. & Fitzgerald, G. F. (1994). Effect of genetically modifying the lactococcal proteolytic system on ripening and flavor development. *Appl. Environ. Microbiol.*, **60**, 4226–33.

Meyer-Barton, E. C., Klein, J. R., Imam, M. & Plapp, R. (1993). Cloning and sequence analysis of the X-Prolyl-dipeptidyl-aminopeptidase gene (pepX) from *Lactobacillus delbrueckii* ssp. *lactis* DSM 7290. *Appl. Microbiol. Biotechnol.*, **40**, 82–9.

Midwinter, R. G. & Pritchard, G. G. (1994). Aminopeptidase N from *Streptococcus salivarius* subsp. *thermophilus* NCDO 573: purification and properties. *J. Appl. Bacteriol.*, **77**, 288–95.

Mierau, I., Tan, P. S. T., Haandrikman, A. J., Mayo, B., Kok, J., Konings, W. N. & Venema, G. (1993). Cloning and sequencing of the gene for a lactococcal endopeptidase, an enzyme with sequence similarity to mammalian enkephalinase. *J. Bacteriol., 175*, 2087–96.

Mierau, I., Haandrikman, A. J., Velterop, O., Tan, P. S. T., Leenhouts, K. J., Konings, W. N., Venema, G. & Kok, J. (1994). Tripeptidase gene (pepT) of *Lactococcus lactis*: molecular cloning and nucleotide sequencing of pepT and construction of a chromosomal deletion mutant. *J. Bacteriol.*, **176**, 2854–61.

Monnet, V., Ley, J. P. & Gonzalez S. (1992). Substrate specificity of the cell envelope-located proteinase of *Lactococcus lactis* subsp. *lactis* NCDO 763. *Int. J. Biochem.*, **24**, 707–18.

Monnet, V. (1994). Methods in Enzymology. Proteolytic enzymes: aspartic, metallo-and other peptidases, ed., A.J. Barret. Academic Press, San Diego, CA. In press.

Monnet, V., Nardi, M., Chopin, A., Chopin, M.-C. & Gripon, J.-C. 1994). Biochemical and genetic characterization of PepF, an oligoendopeptidase from *Lactococcus lactis*. *J. Biol. Chem.*, **269**, 32070–76.

Nardi, M., Chopin, M.-C., Chopin, A., Cals, M.-M. & Gripon, J.-C. (1991). Cloning and DNA sequence analysis of an X-prolyl dipeptidyl peptidase gene from *Lactococcus lactis* subsp. *lactis* NCDO 763. *Appl. Environ. Microbiol.*, **57**, 45–50.

Nowakowski, C. M., Bhowmik, T. & Steele, J. L. (1993). Cloning of peptidase genes from *Lactobacillus helveticus* CNRZ 32. *Appl. Microbiol. Biotechnol.*, **39**, 204–10.

Pritchard, G. G. & Coolbear, T. (1993). The physiology and biochemistry of the proteolytic system in lactic acid bacteria. *FEMS Microbiol. Rev.*, **12**, 179–206.

Reid, J. R., Ng., K. H., Moore, C. H., Coolbear, T. & Pritchard, G. G. (1991). Comparison of bovine β-casein hydrolysis by P_I and P_{III}-type proteinases from *Lactococcus lactis* subsp. *cremoris*. *Appl. Microbiol. Biotechnol.*, **36**, 344–51.

Reid, J. R., Coolbear, T., Pillidge, C. J. & Pritchard, G. G. (1994). Specificity of hydrolysis of bovine κ-casein by cell envelope-associated proteinase from *Lactococcus lactis* strains. *Appl. Environ. Microbiol.*, **60**, 801–6.

Rul, F., Monnet, V. & Gripon, J.-C. (1994). Purification and characterization of a general aminopeptidase (St-PepN) from *Streptococcus salivarius* ssp. *thermophilus* CNRZ 302. *J. Dairy Sci.*, **77**, in press.

Shahbal, S., Hemme, D. & Desmazeaud, M. J. (1991). High cell wall-associated proteinase activity of some *Streptococcus thermophilus* strains (H-strains) correlated with high acidification rate in milk. *Lait*, **71**, 351–7.

Shahbal, S., Hemme, D. & Renault, P. (1993). Characterization of a cell-envelope-associated proteinase activity from *Streptococcus thermophilus* H-strain. *Appl. Environ. Microbiol.*, **59**, 177–82.

Smid, E. J., Driessen, A. J. & Konings, W. N. (1989a). Mechanism and energetics of dipeptide transport in membrane vesicles of *Lactococcus lactis*. *J. Bacteriol.*, **171**, 292–8.

Smid, E. J., Plapp, R. & Konings, W. N. (1989b). Peptide uptake is essential for growth of *Lactococcus lactis* on the milk protein casein. *J. Bacteriol.*, **171**, 6135–40.

Strϕman, P. (1992). Sequence of a gene (lap) encoding a 95.3-kDa aminopeptidase from *Lactococcus lactis* ssp. *cremoris* Wg2. *Gene*, **113**, 107–12.

Tan, P. S. T., van Alen-Boerrigter, I. J., Poolman, B., Seizen, R. J., de Vos, W. M. & Konings, W. N. (1992). Characterization of the *Lactococcus lactis* pepN gene encoding an aminopeptidase homologous to mammalian aminopeptidase N. *FEBS Lett.*, **306**, 9–16.

Tsakalidou, E. & Kalantzopoulos, G. (1992). Purification and partial characterization of an intracellular aminopeptidase from *Streptococcus salivarius* subsp. *thermophilus* strain ACA-DC 114. *J. Appl. Bacteriol.*, **72**, 227–32.

Tynkkynen, S., Buist, G., Kunji, E., Kok, J., Poolman, B., Venema, G. & Haandrikman, A. (1993). Genetic and biochemical characterization of the oligopeptide transport system of *Lactococcus lactis*. *J. Bacteriol.*, **175**, 7523–32.

Van de Guchte, M., Kodde, J., van der Vossen, J. M. B. M., Kok, J. & Venema, G. (1990). Heterologous gene expression in *Lactococcus lactis* subsp. *lactis*: synthesis, secretion, and processing of the *Bacillus subtilis* neutral protease. *Appl. Environ. Microbiol.*, **56**, 2606–11.

Visser, S., Exterkate, F. A., Slangen, K. J. & de Veer, G. J. C. M. (1986). Comparative study of the action of cell wall proteinases from various strains of *Streptococcus cremoris* on bovine α_{s1}-β-and κ-casein. *Appl. Environ. Microbiol.*, **52**, 1162–6.

Visser, S., Robben, A. J. P. M. & Slangen, C. J. (1991). Specificity of a cell envelope-located proteinase (P_{III}-type) from *Lactococcus lactis* subsp. *cremoris* AM1 and its action on bovine β-casein. *Appl. Microbiol. Biotechnol.*, **35**, 477–83.

Vongerichten, K. F., Klein, J. R., Matern, H. & Plapp, R. (1994). Cloning and nucleotide sequence analysis of pepV, a carnosinase gene from *Lactobacillus delbrueckii* subsp. *lactis* DSM 7290, and partial characterization of the enzyme. *Microbiology*, **140**, 2591–600.

Vos, P., Boerrigter, I. J., Buist, G., Haandrikman, A. J., Nijhuis, M., de Reuver, M. B., Siezen, R. J., Venema, G., de Vos, W. M. & Kok, J. (1991). Engineering of the *Lactococcus lactis* proteinase by construction of hybrid enzymes. *Protein Eng.*, **4**, 479–84.